## REMARKS

The foregoing amendments and these remarks are filed in response to the Final Office Action dated August 11, 2009 (the "Office Action"), along with a Request for Continued Examination and a Request for an Extension of Time. Authorization is given to charge the RCE and extension fees to deposit account no. 50-0951.

At the time of the Office Action, claims 98, 119-124, 130, 131, 135-136, 141-143, 145-148, 150-155, and 158 were pending. In the Office Action, objections were raised to claims 119, 146-148, 150 and 157. Claims 146, 148, and 150 were rejected under 35 U.S.C. §112. Claims 98, 119-124, 130, 131, 135, 136, 141-143, 145-148, 150-155, and 158 were rejected under 35 U.S.C. §103(a).

In this response, the objected to and rejected claims have been cancelled. New claims 159-181 have been added. Due to the number of previously filed claims, no additional claim fees are believed to be necessary; however, authorization is given to charge any necessary fees to deposit account no. 50-0951. The new claims are fully supported by the specification and recite alternate subject matter.

### I. Objections to Claims, and Rejections under 35 U.S.C. §112, second paragraph

The examiner has objected to claims 146, 148 and 150 for containing the trade name TEXAS RED without indicating its chemical name. New claim 172 properly recites this trade name and its chemical name. Withdrawal of this objection is therefore appropriate.

### II. Rejection under 35 U.S.C. §103(a)

The claims pending at the time of the last Office Action response were rejected as being unpatentable under 35 U.S.C. §103(a), based upon a combination of Myakishev et al, Nauck et al, Nazarenko et al, Becker et al, and Holland et al. Applicant respectfully submits that the newly submitted claims are patentable over the cited art.

In this regard, Applicant notes that the method of Myakishev does not disclose a combination of two labels formed on target amplification. To the contrary, the combination of two labels is formed in absence of an amplification reaction. The combination of two labels breaks on target amplification, and the two labels are on one primer and not on two primers.

Furthermore, the two universal hairpin energy transfer primers, two allele specific primers and a common reverse primer have been used in each genotyping reaction. In each genotyping reaction, two different targets corresponding to two different alleles (normal and mutated) of a SNP are amplified and for each target amplification one labelled hairpin energy transfer primer and one unlabelled allele specific primer and an unlabelled common reverse primer is used. Two different labeled universal hairpin energy transfer primers are used for two different amplification reactions to amplify two different targets (corresponding to two different alleles) simultaneously.

In Myakishev at page 165, in the paragraph under the caption "Optimization of Assay Condition and Sensitivity," only one labeled primer is used in one amplification reaction, as opposed to two label primers. Applicant notes that the Office Action appears to confuse two labeled sequences of a hairpin primer, which is a single oligonucleotide and contains four different nucleotide sequences, with two labeled primers of the method of claim 159.

At the end of the second paragraph of page 164, Myashivek discloses that the distance between the allele specific and reverse primers is not critical and varied from 7 to 157 bp, meaning that there was no difference in amplification or detection of SNP whether the above-mentioned distance between the allelic primer and the reverse primer was 7 bp or 157 bp. Allele specific primers are not the actual amplification primer that drives the amplification reaction. They are used for adding a non-target tail sequence to the target sequence. The amplification primers are the universal hairpin energy transfer primer hybridizing to the added tail sequence, and the reverse primer. The above distance variation of 7 to 157 bp is between the allele specific primer and the reverse primer and not between the actual amplification primers. The distance between the two actual amplification primers, on the other hand, vary from a minimum 25 – 30 bp to a maximum 175 to 180 bp, where the length of the allelic primers given in table 1 at page 164 varies from 18 nucleotides to 23 nucleotides and the tails are 21 nucleotides long.

Turning to Nauck, this reference does not teach a method of amplifying nucleic acids to give products of 2 bp more than the primer dimer. Two bp is the distance between an anchor probe and a detection probe used in the amplification reaction, and is not the distance between

the two primers of the amplification reaction. The difference between the two primers is 113 bp (the 3' end of one primer is 720 nucleotides away from the transcription start site, and the 3' end of other primer is 607 nucleotides away from the transcription start site), as noted in the second paragraph of the second column of page 1142 of Nauck.

Returning to Myakishev, this reference discloses the use of a hairpin primer, which is known to open in a sluggish manner. Opening of a hairpin primer can be a limiting factor resulting in no variation in amplification with increase in size of the product. This sluggish opening of hairpin primer may be the reason for the disclosed detection limit of 0.4 ng DNA, which is not a very high sensitivity.

Neither Myakishev nor Nauck discloses an amplification product in the size range of 0 – 24 bp more than primer dimer. These references do not address optimization of the distance to maximize the result, as the signal generation occurs by energy transfer between a donor moiety on one primer and an acceptor moiety on another primer. The requirement of energy transfer between donor and acceptor moiety is that the separation of two moieties should be 10–100 Angstrom, which is a separation of 0–28 bp between two moieties and a separation of approximately 0–25 bp between the 3' ends of the two primers in the amplification product. The above effect is observed when such primers are used in amplification a reaction.

Turning now to Nazarenko, this reference teaches the use of labeled primers for quantification of amplified products by FRET. The Office Action asserts that Nazarenko teaches detection of nucleic acid by nucleic acid amplification, use of pair of primers, lengths of primers, donor –acceptor MET moieties and MET distance of 10 – 100 Angstrom, different target acids like genomic or cDNA or mRNA etc.. Taq DNA polymerase, cycling of denauration and annealing steps, labeling of primers with different fluorophore and/or quenchers including internal labeling, different FRET pairs and moieties, semi-nested PCR, and melting of hairpin stem etc. These are all general or common elements or steps of nucleic acid amplification and fluorescence signal generation.

Nazarenko teaches the use of only one labeled primer, and the labeled primer is an energy transfer primer. Each energy transfer primer independently generates a FRET signal is a self-

sufficient signal generating entity with a donor moiety and an acceptor moiety that are in energy transfer relation when there is no amplification. A signal is generated on target amplification through separation of donor and acceptor and disruption of energy transfer. There is no third label moiety that is part of signal generation. Where two hairpin energy transfer primers are used, each generates a signal independent of the other energy transfer primer. There is no interaction between the energy transfer primers, and there is no signal generation through an interaction between a MET moiety of one energy transfer and a MET moiety of another energy transfer primer.

The Office Action cites to column 16, lines 23--26 of Nazarenko, where use of a dual labeled linear energy transfer primer, and an energy transfer primer of triamplification has been mentioned. Notably, in a linear energy transfer primer scenario, a donor and an acceptor are placed on one linear primer and there is energy transfer between the donor and the acceptor where the acceptor quenches the donor emission when there is no target amplification. On target amplification, the linear energy transfer primer gets incorporated into the amplification and a signal is measured after separating donor and acceptor from each other and disrupting the energy transfer of the unincorporated or unutilized energy transfer primers. Jingyue Ju et al in Proc. Natl. Acad. Sci. USA (1995), vol 92 pages 4347 – 4351(entire article particularly last page) and Wang, Y et al in Anal. Chem., (1995), 67, 1197 –1203 have described use of such a primer in DNA sequencing by Sequenase enzyme or PCR cycle sequencing and in PCR. The authors have added separation of donor and acceptor of unutilized primer to measure a signal for nucleic acid detection.

In the energy transfer primer of triamplification, the reverse primer is provided and labeled with a donor and the blocking oligonucleotide, which is complementary to the reverse primer, is provided labeled with an acceptor. The labeled reverse primer and the labeled blocking oligonucleotide form a duplex, and there is energy transfer from donor to acceptor when there is no target amplification. On target amplification the duplex of reverse primer and blocking oligonucleotide get incorporated into the amplification and a signal is measured after separating the donor and acceptor of unincorporated or unutilized duplex energy transfer primer. The 3' end

of the blocking oligonucleotide is capped so that it cannot be extended. Hence the blocking oligonucleotide cannot be a primer of nucleic acid amplification. Here the blocking oligonucleotide acts as a probe or as a part of an energy transfer primer.

In the method of present application, the donor and acceptor moieties are not in one primer, rather the donor is in one primer and the acceptor is in another primer, where the donor and acceptor are separated from each other when there is no amplification, and on target amplification both primers get incorporated into the amplification product so that the two moieties can form a MET/FRET combination and an energy transfer takes place from donor to acceptor resulting in a signal from acceptor. There is no probe for specificity of amplification in the present method. Labeled amplification primers provide the specificity of amplification.

The Office Action also cites to column 20, lines 21–66 of Nazarenko, asserting that it teaches a hairpin primer universal energy transfer primer. Each hairpin universal energy transfer primer is a single primer and is a single oligonucleotide with four different nucleotide sequences in it. First and third sequences are 6–30 nucleotides long each and carry separately a first and a second moiety selected from donor and acceptor moieties and forms the duplex or stem structure of the hairpin because of their sequence complementarities. A second nucleotide sequence 3-20 nucleotides long and a fourth nucleotide sequence 8–40 nucleotides long are single stranded, where the second sequence form a single stranded loop structure and the fourth sequence act as the priming sequence of the hairpin energy transfer primer. All of these four sequences are joined through their 5' and 3' ends to form a single oligonucleotide. There is no third oligonucleotide or third label moiety.

In Table 5, the authors have tabulated nucleotide sequences of two different alleles of a polymorphic target region and two hairpin energy transfer primers for amplifying these two allelic targets. The first hairpin energy transfer amplifies the first allele in association with the common unlabeled reverse primer and the second hairpin primer amplify the second allele in association with the common unlabeled reverse primer. These two allele specific hairpin energy transfer primers are identical except in one base, the allelic base. So there is no labeled third oligonucleotide or third label moiety in Nazarenko.

In Figure 6 of Nazarenko, the authors depict the use of a hairpin energy transfer primer in triamplification in which one hairpin energy transfer forward primer, an unlabeled reverse primer and an unlabeled blocking oligonucleotide which is not a primer, as the 3' end of this oligonucleotide is blocked so that polymerase cannot extend this oligonucleotide. Thus, there is no third label, and only one primer is labeled.

In Figure 7 of Nazarenko, there is disclosed a triamplification reaction in which the forward primer (F) is provided unlabeled, the reverse primer (R) is provided labeled with a donor moiety (D) near 3' end and a blocking oligonucleotide (BL) labeled with an acceptor moiety (A) near 5' end, a phosphate modification at 5' end for ligation purpose and with a biotin molecule at 3' end to block the extension of the blocking oligonucleotide by the polymerase.

In the Office Action, the above-noted labeled first nucleotide sequence and labeled third nucleotide sequence of hairpin energy transfer primer may be mistaken to be a the first labeled primer and third labeled oligonucleotide as claimed. Notably, however, there is an additional second labeled oligonucleotide primer in the above claims of the present method. There are three label moieties in these claims, whereas in Nazarenko et al there are only two label moieties. The Office Action appears to incorrectly assert that the labels of the above first and third sequences of the first hairpin energy transfer primer for amplifying first allele as the first labeled primer and third labeled oligonucleotide of the above claims of the present method, and the second hairpin energy transfer primer for amplifying the second allele as the second labelled primer of the present. But, these two hairpin primers are not involved in amplifying the same target. To the contrary, they amplify two different targets (corresponding to two different alleles) in association with a common unlabelled reverse primer. There is neither any interaction between the two hairpin energy transfer primers nor there is any energy transfer interaction between the labels of first and second hairpin energy transfer primers of Nazarenko. In addition, only two label moieties fluorescein and DABCYL have been used in all labeled energy transfer primers. There is no third label moiety. The first and second labeled primers of the above claims of the present method are involved in amplifying one target sequence, and in the amplification product there is energy transfer interaction between the two label moieties on the first and second primers. In the

above claims of the method of present application there are three labels and three labeled oligonucleotides of which two labeled oligonucleotides are two primers and there is energy transfer between the labels of two primers and a signal is generated from such energy transfer. The third oligonucleotide and the third label are used for reducing the background emission or noise and not for signal generation. Hence there is a basic difference in the signal generation of the present method with respect to Nazarenko.

Turning to Becker, this reference discloses two unlabeled primers that are used for amplifying a target sequence and a FRET labeled probe (labeled with a donor at one end and an acceptor at the other end, which are in energy transfer or FRET relation) is nested between the two primers. The FRET labeled probe does not amplify any segment as it happens in nested nucleic acid amplification. In contrast, in the method of claim 159, both primers (forward and reverse) of nucleic acid amplification are labeled, one with a donor moiety and the other with an acceptor moiety. In addition, there is no energy transfer between the donor and acceptor when there is no target amplification. There cannot be any complementarities between two primers. On target amplification, there is an energy transfer from donor to acceptor and acceptor emission is measured.

In sum, detection of a target nucleic acid by nucleic acid amplification is a highly sensitive method where the target nucleic acid is amplified millions to billions of times. Being a highly sensitive method, it has associated problems of non-specific amplification from non-specific annealing of primers and primer dimer formation, which results in amplification reaction failure. Non-specific amplification products and primer dimer are the two major problems of nucleic acid amplification. Primer dimer formation is a major problem when a few target sequences are present in a sample. Whenever there are less than 10,000 copies of target nucleic acid in an amplification reaction, primer dimer starts forming and primer dimer formation keeps on increasing as the copies of the target nucleic acid in the amplification reaction reduces further. It has been difficult in the field to avoid formation of primer dimer. Even with the best possible primer pair designing, and the best possible optimization of an amplification reaction, it has not been possible to avoid primer dimer formation. Moreover, whenever there is inhibition in the

amplification reaction due to inhibitor in the sample or any other reason, primer dimer formation increases.

The solution to the problem of detection of specific amplification product in the presence of non-specific amplification product and primer dimer has been separation of the specific amplification product from the non-specific amplification product and primer dimer and subsequent detection of the specific product by a signal generation protocol, or separation and hybridization of a probe specific for the target sequence and a suitable signal generation protocol. When a FRET based signal generation is used in combination with nucleic acid amplification, no separation is required for nucleic acid detection or quantitation. In this regard, mainly a probe with suitable signaling moiety or moieties and a signal generation protocol has been used. For this non-separation base detection an oligonucleotide probe labeled with a fluorophore and a quencher at two ends and a polymerase with 5' exonuclease activity has been used for signal generation where the fluorophore and the quencher get separated due to 5' exonuclease hydrolysis of the probe if it is hybridized to the target, as described by Holland et al Proc. Natl. Acad. Sci. USA, 88,7276-80, 1991, Lee et al Nucleic Acid Res. 21, 3761-66, 1993 (acknowledged in the specification). There is a problem of sluggish amplification due to slow hybridization of the probe and obstruction by the probe to the polymerase extension which affect sensitivity, and moreover the fluorphore and quencher are at two ends of the probe where because of longer distance between the two moieties quenching is not efficient. Furthermore, the polymerase may displace the probe before a signal is generated and the probe can hybridize non-specifically resulting in noise. This results in low signal to noise ratio. In another case (Tyagi and Kramer, 1996, Nature Biotech. 14, 303 -309 and US patent 5,312,728)(as acknowledged in the specification), a hair-pin probe labeled with a fluorophore and a quencher at two ends of the stem of the hair-pin probe, which gives a signal when it hybridizes to the specific target sequence through separation of the fluorophore and quencher have been used in nucleic acid amplification. The problem of the use of hairpin probe is sluggish opening of the hairpin probe, which affects the sensitivity of target detection. In addition, there are problems associated with hybridization of a probe. Furthermore, designing a suitable hairpin probe is not always possible. There are

methods where oligonucletides labeled with signaling moieties are ligated resulting in signal generation. In addition, there are methods based on use of a primer labelled with one fluorophore and a probe labelled with another fluorophore, where a signal is generated from the said two moieties on the primer and the probe on nucleic acid amplification and probe hybridization. There are methods where two probes labelled separately with two fluorophore are used and a signal is generated on hybridization of two probes. In this category are the methods of US patent 6,140,054 to Witter et al (acknowledged in the specification) and Nauck.

Use of a probe renders the amplification reaction sluggish and less efficient, resulting in less sensitivity. There are methods in which a primer suitably labeled with a signaling moiety for a signal generation is used. In this category falls the disclosures of Nazarenko and Myakishev. In addition, Nazarenko and Myakishev use only one of the two amplification primers as a labeled primer. Nauck uses both amplification primers as unlabeled primer and uses two probes each labeled separately with a donor or an acceptor. Becker and Holland use both amplification primers as unlabeled primer and use a single probe labeled with two labels at two ends.

No method has used two primers of nucleic acid amplification as a labeled primer. Notably, there is a major hurdle for the use of a donor labeled primer and an acceptor labeled primer for signal generation to detect a nucleic acid by amplification. The major hurdle is that the two primers of an amplification reaction labeled with a donor or an acceptor moiety respectively cannot be used in a nucleic acid amplification reaction, as primer dimer formed from such primers would generate a FRET signal more intense than the specific amplification product generates. It is known that FRET signal is inversely proportional to six power of the distance between donor and acceptor as a result it reduces drastically with increase in distance between the donor and acceptor moieties. Moreover, it has been known that it is very difficult or impossible to avoid primer dimer formation. Therefore, even a skilled person in the art would not think of using such labeled primers for nucleic acid analysis. Therefore, use of such a labeled primer for FRET signal generation cannot be anticipated or motivated from the disclosure of any of the documents cited. Before using two labeled primers for signal generation, one has to find a very good solution to the primer dimer problem, which previously was not possible.

The present application teaches that two primers labeled with a donor moiety or an acceptor moiety do not form primer dimer. For forming a primer dimer the polymerase has to extend one labeled primer over another labeled primer. For such an extension, the polymerase encounters a fluorophore moiety in a short distance while extending the other labelled primer. Such an encounter results in inhibition or prevention of such extension and, thus, inhibition or prevention of primer dimer formation. This is also unexpected according to prior knowledge as nucleotides labeled with fluorescent moiety or moieties have been incorporated into nucleic acid in nucleic acid synthesis by polymerase based extension as well as in PCR amplification incorporating fluorescent moiety labeled nucleotides in the amplification product. Further, such use of two labeled primers requires the donor and acceptor to be within 10-100 Angstrom, which is the distance limit of FRET. This requirement needs amplification of an amplification product in which 3' ends of two primers remain separated by 0–25 bp. Applicant also unexpectedly determined that while amplifying amplification product of such size that primer dimer formation is much reduced even when there are a few targets in the amplification reaction. The yield of the amplification product is very high in comparison to bigger size amplification products. Also non-specific amplification products are not formed due to such selection of amplification product. Further, because of the primer dimer problem, amplifying amplification product of such small size has been avoided as it becomes difficult to distinguish an amplification product from primer dimer product while analyzing the amplification product by gel electrophoresis. Because of this, amplification of an amplification product in this size region is avoided.

Regarding the Office Action's citation of Chetverin et al (WO 1993/17126) and US 6,210,897 to Andersson. Chetverin teaches attaching an oligonucleotide probe to a solid surface through covalent linking and hybridizing a target sequence to that probe and measuring a signal. Andersson et al teaches attaching an oligonucleotide probe to a solid surface through covalent linking and hybridizing a target sequence to the linked probe and ligating this linked probe to another probe, which also hybridizes to the same target. Covalent linking or covalent attachment to a solid surface has been known for a very long time. It is also well known that the rate of reaction of any reaction on a solid surface is slower than that in solution phase. As in

hybridization of a probe to a target sequence, no enzymatic reaction takes place and hence the hybridization reaction can be continued for a very long time so that a quantitative hybridization can be achieved. Hybridized probe can later be reacted with enzymes. On the other hand, in the case of nucleic acid amplification enzymatic reaction of a polymerase, repeated cycling of denaturation, annealing and extension is also involved. If the amplification reaction is carried out in solid phase, each step in each cycle would take longer. As a result, the polymerase would lose its enzymatic activity with progress of the reaction as the enzyme has a definite lifetime. Because of this, amplification reaction would be inefficient resulting in lower sensitivity.

**III. Conclusion**

Applicant has made every effort to present claims that distinguish over the prior art. All claims are believed to be in condition for allowance. Nevertheless, Applicant invites the Examiner to call the undersigned if it is believed that a telephonic interview would expedite the prosecution of the application to an allowance. In view of the foregoing remarks, Applicant respectfully requests reconsideration and prompt allowance of the pending claims.

Respectfully submitted,

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